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# Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America

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**ABSTRACT** **The silver-haired bat variant of rabies virus (SHBRV) has been identified as the etiological agent of a number of recent human rabies cases in the United States that are unusual in not having been associated with any known history of conventional exposure. Comparison of the different biological and biochemical properties of isolates of this virus with those of a coyote street rabies virus (COSRV) revealed that there are unique features associated with SHBRV. *In vitro* studies showed that, while the susceptibility of neuroblastoma cells to infection by both viruses was similar, the infectivity of SHBRV was much higher than that of COSRV in fibroblasts (BHK-21) and epithelial cells (MA-104), particularly when these cells were kept at 34°C. At this temperature, low pH-dependent fusion and cell-to-cell spread of virus is seen in BHK-21 cells infected with SHBRV but not with COSRV. It appears that SHBRV may possess an unique cellular tropism and the ability to replicate at lower temperature, allowing a more effective local replication in the dermis. This hypothesis is supported by *in vivo* results which showed that while SHBRV is less neurovirulent than COSRV when administered via the intramuscular or intranasal routes, both viruses are equally neuroinvasive if injected intracranially or intradermally. Consistent with the above findings, the amino acid sequences of the glycoproteins of SHBRV and COSRV were found to have substantial differences, particularly in the region that contains the putative toxic loop, which are reflected in marked differences in their antigenic composition. Nevertheless, an experimental rabies vaccine based on the Pittman Moore vaccine strain protected mice equally well from lethal doses of SHBRV and COSRV, suggesting that currently used vaccines should be effective in the postexposure prophylaxis of rabies due to SHBRV.**

Recent trends of human rabies in the United States indicate that a new form of rabies may be emerging. In the 14 years prior to 1994, there was, on average, less than one indigenous case reported per annum in the United States (1–3). However, in 1994 and 1995, nine more indigenous cases of human rabies were reported. The etiological agent involved in five of these cases was identified as a rare variant of rabies virus associated with silver-haired bats (4–6). The prevalence of the silver-haired bat variant in recent human rabies cases is even more puzzling because the host is relatively uncommon, representing <8% of the total number of rabid bats identified in the United States, and has a solitary life style (1). Also, unlike classical rabies, these recent human cases have not been definitively related to any known exposure such as animal bites, scratches, or contact with aerosols (1, 7, 8). This has contributed to the fact that rabies was not diagnosed ante mortem in most of these victims (1–3). The unknown yet undoubtedly atypical mode of the transmission together with the rarity of the natural host has led us to hypothesize that the silver-haired bat variant of rabies virus may have unique biological properties that enhance its transmissibility to humans.

It is well known that common street rabies virus strains, such as canine variants, are usually transmitted by bite and are highly neurotropic. Viral replication is almost exclusively restricted to neuronal cells (9). Nevertheless, rabies virus may replicate at the inoculation site (10, 11). However, the contribution of local replication at the inoculation site to transmission is unknown. In this regard there has been much speculation concerning the existence of particular rabies virus receptors. Experimental data obtained to date provide only suggestive evidence that the nicotinic acetylcholine receptor may serve as the rabies virus attachment site (12). Alternatively there is some evidence that other cellular factors may be responsible for the neurotropism of rabies virus (13). Several studies clearly indicate that the glycoprotein (G) of rabies virus plays an essential role in its neuropathogenesis. For example, the use of antigenic variants representing operationally defined antigenic sites on the G protein of several fixed rabies virus strains showed that the neuropathogenicity of the virus correlates with the presence of a determinant located within antigenic site III (14, 15). It is noteworthy in this regard that recent findings suggest that the fusogenic activity of the virulent type G protein is responsible for the more efficient spread of virulent virus in the central nervous system (16).

To obtain insight into possible mechanisms involved in the pathogenesis of rabies caused by the silver-haired bat rabies virus variant (SHBRV), we have characterized SHBRV biologically and biochemically by (*i*) examining its neuroinvasiveness in mice, (*ii*) investigating its ability to infect neuronal and nonneuronal cells in culture and to induce fusion in such cells, and (*iii*) analyzing the chemical and antigenic structure of the SHBRV G protein. The results of these investigations support our hypothesis that the SHBRV variant may have unique properties that directly relate to its infectivity, and that rabies mediated by this virus may have its genesis in an atypical infection.

## MATERIALS AND METHODS

**Cells, Viruses, and Antigens.** Monolayer cultures of BHK-21 clone 13 cells, NA neuroblastoma cells of A/J mouse origin, and MA-104 epithelial cells of nonhuman primate origin were grown at 37°C in Eagle's minimum essential medium. SHBRV was obtained from the brain of a naturally infected human

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*Abbreviations*: SHBRV, silver-haired bat rabies virus; COSRV, coyote street rabies virus; G, glycoprotein; βPL, beta-propiolactone; ffu, focus forming units; moi, multiplicity of infectivity; RT–PCR, reverse transcriptase–polymerase chain reaction; mAb, monoclonal antibody; $LD_{50}$, 50% lethal dose; $ED_{50}$, 50% effective dose; PM, Pittman Moore.

*Data deposition*: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U452946 and U452947).

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from California (2) and a coyote street rabies virus variant (COSRV) was obtained from the salivary glands of a naturally infected Texas coyote (17). A stock of each virus [20% brain suspension in phosphate-buffered saline (PBS)] was prepared following a single passage of the primary isolates in suckling mouse brains.

The Pittman Moore (PM) rabies vaccine strain was propagated in BHK-21 cell monolayers and purified as described (18). The purified virus was suspended in PBS, inactivated with β-propiolactone (βPL), and adjusted to a protein concentration of 100 μg/ml.

**Virus Infectivity Assay.** Monolayers of NA, BHK-21, or MA-104 cells in 96-well plates were infected with 50 μl of virus at serial 10-fold dilutions and incubated for 1 hr to allow virus adsorption. The virus inoculum was then removed, the cultures were replenished with 100 μl culture medium, and then they were incubated at 37°C or 34°C. Forty-eight hours after infection the cells were fixed in 80% acetone and subjected to the fluorescent staining technique (19); foci were counted by using a fluorescent microscope. All titrations were carried out in triplicate.

**Production of Infectious Virus.** NA, BHK-21, or MA-104 cells were infected with SHBRV or COSRV at a multiplicity of infection (moi) of 5 focus forming units (ffu) per cell. After infection, cells were incubated at either 37°C or 34°C. At 24-hr intervals, culture medium was removed and cells were replenished with new culture medium. The amount of virus released into the culture medium was determined by virus titration in NA cells as described above.

**Virus Cell-Spread Assay.** NA cells or BHK-21 cells were cultured in 24-well plates. SHBRV or COSRV (200 μl) were added to the confluent monolayer at a moi of 0.1 and cells were incubated for 2 hr at 37°C. The virus inoculum was removed and 100 μl of culture medium containing a virus neutralizing monoclonal antibody (mAb 523, neutralization titer: 1:1000) was added to each well. Seventy-two hours after infection, cells were fixed in 80% acetone, and infected cells were identified by the direct fluorescent staining technique (19).

**Low pH-Dependent Cell Fusion.** NA or BHK-21 cells infected with a moi of 5 ffu of either SHBRV or COSRV were incubated at 37°C for 24 hr and then at 34°C for 3 weeks. During this period, cells were split once a week. Persistently infected cells were trypsinized, seeded into 24-well plates, and incubated at 37°C until the cell monolayers were about 60% confluent. Then plates were incubated for 18 hr at either 37°C or 34°C. The cells were rinsed with fusion medium [10 mM $Na_2HPO_4$/10 mM $NaH_2PO_4$/150 mM NaCl/10 mM 2-(N-morpholino) ethanesulfonic acid], adjusted to a pH range from 5.2 to 6.0, then incubated for 1 min at room temperature with pH-adjusted fusion medium. After removal of the fusion medium, cell cultures were replenished with medium. Following incubation for 16 hr at either 37°C or 34°C, the cells were fixed with 80% acetone, and the percentage of fused cells was determined using a light microscope, as described (16).

**Extraction of RNA.** Total RNA was isolated from virus-infected mouse brains according to the manufacturer's manual for the RNAzol B method (Biotecx Laboratories, Houston). The RNA was subjected to reverse transcriptase–polymerase chain reaction (RT–PCR) to obtain the cDNAs of rabies virus G protein gene.

**RT–PCR, cDNA Cloning, and Sequencing of the Rabies Virus G Protein Gene.** RT reactions were performed at 42°C for 1 hr using avian myeloblastosis virus reverse transcriptase (Promega) as previously described (20). Primer COSRV-3, 5′-ATCAGGATCCTGGATCGTTGAAAGGA-3′, which was used previously for cloning the G protein gene of a canine street rabies virus (21), was initially used for reverse transcription. A portion of the RT product was subjected to PCR amplification by using the primers COSRV-5 (5′-TCCCGAATTCGACTCAAGGAAAGATG-3′) (21) and COSRV-3. Amplification was carried out for 40 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 90 sec, and polymerization at 72°C for 3 min with a *Taq* DNA polymerase (Promega), as described (20). Under these conditions, the cDNA of the G protein gene of COSRV was amplified, giving a specific PCR product (approximately 1.8 kb in length), but that of SHBRV was not. We therefore constructed two additional primers, bat 1 5′-T(C/T)ACAGTC(T/C)(G/A)(G/A)TCTC-3′ [T(C/T) corresponds to the sequences around the stop codon]; and bat 2 5′-GTTGAGATG(A/G)(T/C)TA(A/G)TGAA-3′ which corresponds to a 140-nucleotide region downstream of the stop codon of the conserved 3′ noncoding region of rabies G protein gene as reported by Ravkov *et al.* (22). Primer bat 2 was used for the RT reaction of RNA extracted from SHBRV-infected cells. PCRs were performed with primers COSRV-5 and bat 2. Because the PCR products obtained with these primers contained many DNA fragments, a portion of the products was subjected to further PCR using primers COSRV-5 and bat 1 to obtain a specific 1.6-kb DNA product. The individual PCR products were cloned into the pCRII vector (Invitrogen), and the recombinant plasmids were sequenced by use of the AmpliTaq cycle sequencing kit (Perkin–Elmer) and several synthetic oligonucleotides as primers.

**Antigenic Analysis of the G Protein.** The G proteins of SHBRV and COSRV were characterized by assessing the virus-neutralizing activity of each of 37 G protein-specific mAbs against the two viruses. The neutralization test was performed as described (19). A reduction in viral titer >100 infection units in the presence of a mAb was considered as a positive result.

**Pathogenicity Studies in Mice.** Five- to 6-week-old female ICR mice (Harlan–Sprague–Dawley) were used in these experiments. Groups of 10 mice were inoculated intracranially, intradermally by injection into the skin of the foot pad, intramuscularly by injection into the gastrocnemius muscle, or intranasally with 25 μl of each of the four 5-fold dilutions prepared from the SHBRV and COSRV stocks. The animals were observed for 4 weeks and the 50% lethal dose ($LD_{50}$) was calculated as described (23).

**Immunization and Virus Challenge.** Groups of 10-week-old ICR mice (Harlan–Sprague–Dawley) were inoculated with two intraperitoneal injections (0.1 ml) containing 5, 1, 0.4, and 0.08 μg of βPL-inactivated PM virus, 7 days apart. Ten days after the second immunization, the mice were challenged by intracranial inoculation of 100 mouse $LD_{50}$ of SHBRV and COSRV and then observed for a minimum period of 4 weeks. The 50% effective dose ($ED_{50}$) of the vaccine was calculated as described (24).

## RESULTS

**Pathogenicity of SHBRV and COSRV in Mice.** To examine the possibility that infection with the bat rabies virus variant involves pathogenic mechanisms that are different from those of other street rabies viruses, we first compared the $LD_{50}$ values of SHBRV and COSRV, following different routes of inoculation. Although the intracranial $LD_{50}$ values of both viruses were identical, roughly 10 times more SHBRV than COSRV was necessary to cause a fatal rabies virus infection in 50% of mice inoculated by the intramuscular route (Table 1). A similar difference between the infectivity of the two viruses was seen after intranasal infection. Although this route of inoculation resulted in the lowest $LD_{50}$ obtained for COSRV infection, a significant proportion of the mice died, whereas none of the animals receiving SHBRV via this route succumbed to the infection. In contrast to the other peripheral routes of inoculation, similar $LD_{50}$ values were obtained for both virus preparations after intradermal inoculation.

**Cell Tropism, Virus Production, and Virus Spread in Neuronal and Nonneuronal Cells.** To investigate whether the

Table 1. Pathogenicity of SHBRV and COSRV in mice

| Route of infection | SHBRV | | COSRV | |
|---|---|---|---|---|
| | $LD_{50}$* | Relative pathogenicity† | $LD_{50}$* | Relative pathogenicity† |
| I.C. | $10^{-4.17}$ | 1.0 | $10^{-4.17}$ | 1.0 |
| I.N. | $\ll 10^{0}$ | $\ll 0.00007$ | $10^{-0.65}$ | 0.0003 |
| I.M. | $10^{-0.70}$ | 0.0003 | $10^{-1.69}$ | 0.0033 |
| I.D. | $10^{-1.08}$ | 0.0008 | $10^{-1.00}$ | 0.0007 |

*Groups of 10 female ICR mice were inoculated with serial 5-fold dilutions of SHBRV or COSRV stocks prepared from infected suckling mouse brain. $LD_{50}$ was calculated by the described method (23).

†Relative pathogenicity is the quotient of the I.C. (intracranial), I.M. (intramuscular), I.N. (intranasal), or I.D. (intradermal) $LD_{50}$ and the I.C. $LD_{50}$.

tissue tropism of the SHBRV might differ from that of other street viruses, we compared the susceptibility of neuronal and nonneuronal cells in culture to infection by SHBRV and COSRV. Following the addition of 10-fold dilutions of the SHBV and COSRV stocks, NA, BHK-21, or MA-104 cells were incubated for 48 hr at 37°C, the optimal temperature for growth of the cells, or at 34°C, a suboptimal temperature where cell metabolism is reduced. Virus titers were then determined by the fluorescent focus assay. In NA cells, similar titers of both viruses were obtained and incubation temperature had no (SHBRV) or little (COSRV) effect on virus titers (Table 2). In contrast, the titers of SHBRV obtained in BHK-21 or MA-104 cells were 100–1000-fold or 10–100-fold higher, respectively, than those of COSRV. Furthermore, as shown in Table 2, 40 times greater SHBRV titers were detected when BHK-21 or MA-104 cells were incubated at 34°C instead of 37°C. The incubation temperature had no significant effect on COSRV titers in these cells.

To examine whether SHBRV and COSRV not only differ in their ability to infect but also to replicate in neuronal and nonneuronal cells, we infected NA, BHK-21, and MA-104 cells at a moi of 5 and measured newly synthesized virus every 24 hr for 5 days (Fig. 1). While NA cells produced slightly less SHBRV than COSRV, BHK-21 and MA-104 cells produced, on average, more than a 1000-fold more SHBRV than COSRV at both 34°C and 37°C. However, in contrast to its marked effect on infectivity, incubation temperature had a less clear effect on SHBRV production in BHK-21 or MA-104 cells.

Since virus spread from cell to cell is a characteristic feature of rabies virus infection (25), we examined the spread of virus in NA and BHK-21 cells cultured in the presence of virus-

Table 2. Virus titers of SHBRV and COSRV stocks in NA, BHK-21, and MA-104 cells

| Cell | Temperature, °C | Virus titer | |
|---|---|---|---|
| | | SHBRV | COSRV |
| NA | 37 | $5 \times 10^{6}$ | $1.5 \times 10^{7}$ |
| | 34 | $5 \times 10^{6}$ | $3.5 \times 10^{6}$ |
| BHK-21 | 37 | $1.5 \times 10^{5}$ | $2 \times 10^{3}$ |
| | 34 | $2 \times 10^{6}$ | $1.5 \times 10^{3}$ |
| MA-104 | 37 | $1 \times 10^{4}$ | $1.5 \times 10^{3}$ |
| | 34 | $4 \times 10^{5}$ | $2 \times 10^{3}$ |

neutralizing antibody to prevent infection by virus in the medium. While no differences in the spread of the two viruses were seen in NA cells, only SHBRV was found to spread in BHK-21 cells and only at 34°C (data not shown).

**Low pH-Induced Fusion in SHBRV and COSRV Infected Cells.** Because it is likely that the ability of rabies virus to induce cell fusion is associated with its ability to infect cells and to spread from cell to cell, features that we have found to be different between SHBRV and COSRV, we next studied the fusogenic properties of these viruses in NA and BHK-21 cells (Fig. 2). Fig. 2*A* shows that fusion can be induced in NA cells infected with either SHBRV or COSRV at a pH range between 5.2 and 5.6. Fusion activity of SHBRV- and COSRV-infected NA cells appeared to be somewhat higher at 34°C than at 37°C. In contrast, low pH catalyzed fusion could only be observed in SHBRV-infected BHK-21 cells at 34°C but not at 37°C and no fusion could be induced in COSRV-infected BHK-21 cells at either 37°C or 34°C.

**Comparison of the Chemical Structure and Antigenic Properties of the G Proteins of SHBRV and COSRV.** Because low pH-induced fusion is mediated by the G protein (26), we analyzed the amino acid sequence and antigenic structure of the G proteins of SHBRV and COSRV. The homology of the amino acid sequences of the full-length G proteins of SHBRV and COSRV is about 86%, and comparison of the ectodomains of both proteins revealed a 88% identity (Fig. 3). However, certain regions of the ectodomain show greater diversity than others, such as a sequence of 27 amino acids (residues 181–207) which includes the putative toxic loop. Most notably, the cysteine residue at position 207 conserved in the G protein of all strains sequenced so far is replaced by tryptophan. Not surprisingly, the carbohydrate acceptor site at Asp-319 as well as the Arg-333, a critical residue for rabies virus pathogenicity, are conserved in both G proteins.

The diversity between the amino acid sequences of the G proteins conforms with the differences in the antigenic char-

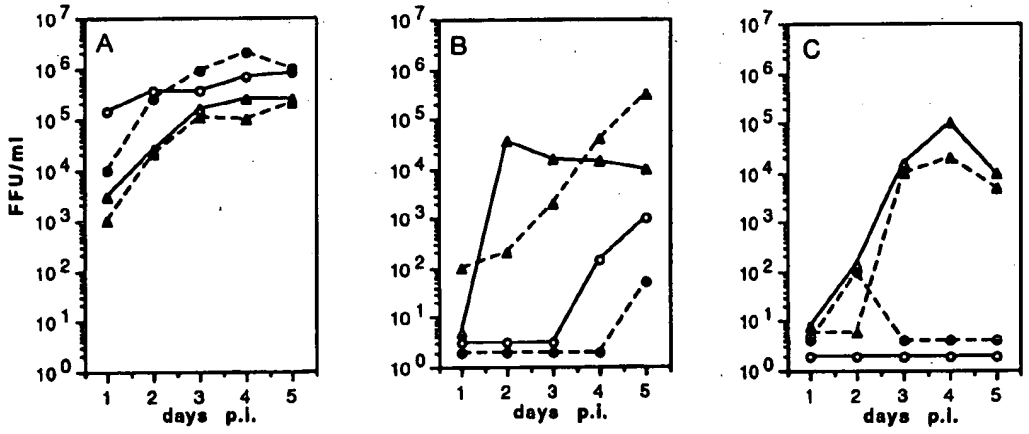


FIG. 1. Virus production in cell cultures. NA (*A*), BHK-21 (*B*), and MA-104 (*C*) cells were infected with SHBRV (triangles) or COSRV (circles) at an moi of 5 ffu and incubated at either 34°C (dotted line) or 37°C (solid line). The culture supernatant was harvested every 24 hr for 5 days, and virus titers were determined as described.

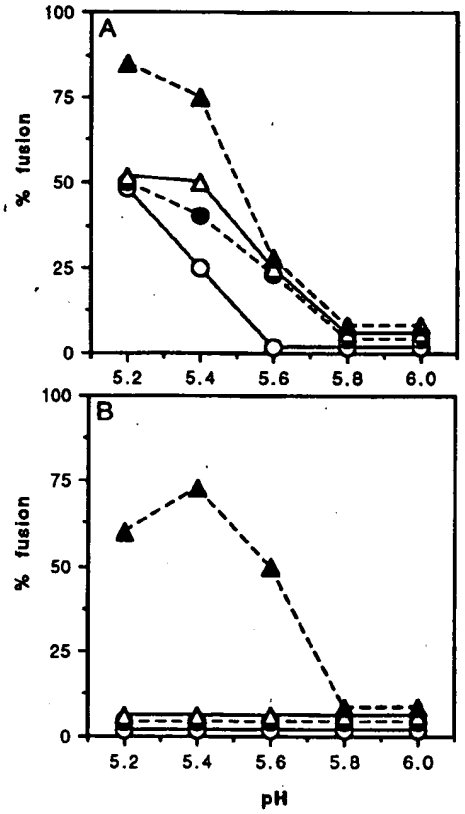


FIG. 2. Low pH-dependent fusion. NA (*A*) or BHK-21 (*B*) cells persistently infected with either SHBRV (triangles) or COSRV (circles) were treated with fusion medium at indicated pH, incubated for 16 hr at 37°C (solid line, open symbols) or at 34°C (dotted line, closed symbols), and fixed with 80% acetone. The percentage of fused cells was determined microscopically.

acteristics of the two virus strains. Antigenic analysis with a panel of 37 G protein-specific mAbs revealed numerous differences among the two virus strains (Fig. 4). Results obtained with these mAbs in virus neutralization indicate that both viruses share only 65% of the determinants. The greatest antigenic differences were found in antigenic site IIIB. From the 11 mAbs in our possession that recognize epitopes located in site IIIB, only 6 (55%) were able to neutralize both virus strains.

The G-protein is the major target of virus-neutralizing antibodies, which are generally considered to be the primary effectors in immune defense against rabies (27). Because of the antigenic differences we observed between the G proteins of SHBRV and COSRV, we believed that it was important to ascertain whether or not conventional rabies vaccines can confer protection against these viruses. Table 3 shows that immunization with a vaccine prepared from the PM strain of rabies virus, which is one of the most frequently used vaccine strains, protected against intracranial challenge infection with SHBRV and COSRV. The potency of the vaccine was equivalent in both cases.

## DISCUSSION

As one element of our analyses of the antigenic structure of the rabies virus variants SHBRV and COSRV we performed protection experiments in mice using an experimental vaccine based on the PM vaccine strain of the virus, which is widely used in the preparation of human vaccines. This vaccine protected mice equally well from infection with lethal doses of

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          |-signal  peptide-|
SHBRV  -19:MIPQALQFVPLLIPSLCFGKFPIYTIPDKLGPWSPIDIHHLSCP
COSRV  -19:.V....L...I.VF.:.......H.....................

SHBRV   26:NNLVAEDEGCTSLSGFSYMELKVGYISAIKVNGFTCTGVVTEAET
COSRV   26:....V......N..................E.A.............

SHBRV   71:YTNFVGYVTTTFKRKHFRPMPDACRAAHDWKMAGDPRYEDSLQNP
COSRV   71:...S.....A..R......T.....S.YN..........E..H..

SHBRV  116:YPDYHWLRTVKTTKESLVIISPSVADLDPYDKSLHSRVFPSGKCL
COSRV  116:.A........R....A.........................I...S

SHBRV  161:GITVSSTYCSTNHDYTIWMPVEARLGTSCDIFTNSKGKKASKGGR
COSRV  161:.....................ENP....W....V..R..R....SK

SHBRV  206:TWGFVDERGLYKSLKGACKLKLCGVPGLRLMDGTWVSIQTSDDIK
COSRV  206:IC.......................L..........AM...EET.

SHBRV  251:WCPPDQLVNLHDFHSDEIEHLVVEELIKKREGCLDALESIMTTKS
COSRV  251:..............R............V....E.............

SHBRV  296:VSFRRLSHLRKLVPGFGKAYTIFNNTLMEADAHYKSVRTWNEVIP
COSRV  296:.......................K.S..............I..

SHBRV  341:SKGCLKVGGRCHPPVNGVFFNGIILGPDGNVLIPEMQSSLLQQHM
COSRV  341:.....R.......H..............DH..............

SHBRV  386:ELLESSVIPLTHPLADPSTVFKDGDEAEDFVEVHLPDVHKQVSEI
COSRV  386:..........M...G.......E...................GV

                    |transmembrane domain|--------------
SHBRV  431:DLGLPSWGKYLLMSAGVLATLILAIFLITCCRRANRTESTQRGRR
COSRV  431:.....N....V.......IS.M.L...M.....V..P.....SPG

          cytoplasmic  domain----------|
SHBRV  476:ESGGKVSVAPQNGKIISSWELYKSGSETGL
COSRV  476:GA.R....TS.S..V.....S....G..R.
```

FIG. 3. Amino acid sequences of rabies virus G proteins of SHBRV and COSRV. Total RNA was isolated from virus-infected suckling mouse brains and subjected to PCR cloning and sequencing as described. Dots indicate conserved sequences between the two viruses. Underlined are the putative glycosylation signals at positions 37–319. The line above positions 181–207 represents a highly variable region. Asterisk denotes the arginine residue at position 333.

SHBRV and COSRV, suggesting that most of the currently used rabies vaccines, in addition to their proven usefulness for people exposed to other rabies virus strains, will also be effective in the pre- and postexposure prophylaxis of rabies caused by the SHBRV variant.

Although the G proteins of various rabies virus strains clearly possess sufficient antigenic similarity to be cross-protective, our analysis of SHBRV and COSRV with a panel of mAbs demonstrated marked differences in their G protein antigenic structure. Sequence analysis also revealed major differences in the structure of the G proteins of SHBRV and COSRV. SHBRV and COSRV share only 88% homology in the ectodomains of their G proteins, compared to the 94–95% homology shared between COSRV and two dog strains, one isolated in France (21) and the other in China (28). Of particular interest is our finding that the greatest diversity in the amino acid sequence between SHBRV and COSRV is located in a stretch of 27 amino acids which contains the putative attachment site for the acetylcholine receptor (29). Variations in the structure of this and other regions of the ectodomain of the rabies G protein are likely to be reflected in changes in the biological properties of the virus, such as tropism and fusogenic activity.

When SHBRV is inoculated directly into the brain, it is as infectious as COSRV, demonstrating that these viruses evidently behave similarly once in the central nervous system. However, SHBRV is less neuroinvasive than COSRV when administered intranasally or intramuscularly, the latter being

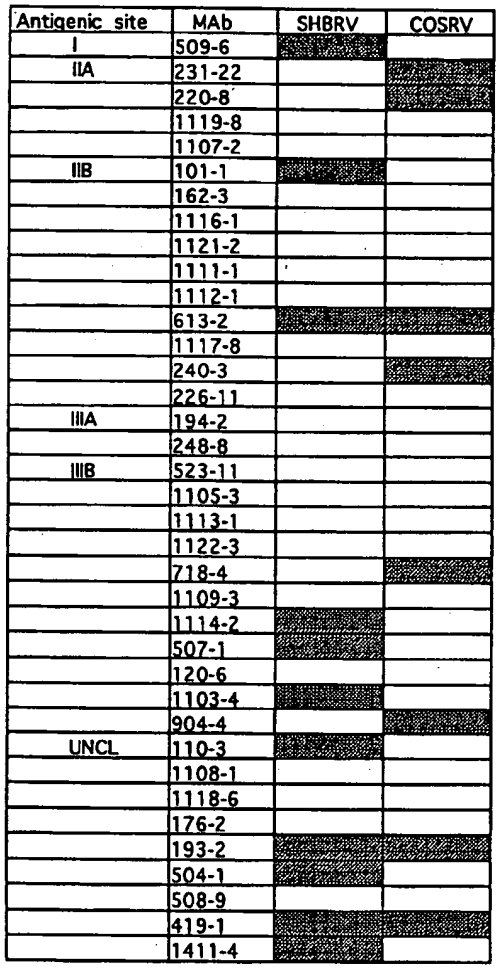


FIG. 4. Antigenic analysis of the G protein. Rabies virus SHBRV and COSRV were subjected to neutralization with a panel of G protein-specific mAbs as described (18). Open boxes represent virus neutralization index > 100; shadowed boxes represent virus neutralization index < 100.

the experimental equivalent of the conventional mode of transmission of rabies virus. In contrast to the relatively weak infectivity of SHBRV administered via other peripheral routes, SHBRV and COSRV appear equally as neuroinvasive when injected intradermally. We believe that the logical conclusion that can be drawn from these surprising findings is that SHBRV possesses some attributes that gives it a selective advantage over COSRV when replicating in the periphery, such as the dermis, and that this was most likely to be a reflection of some difference in cell tropism.

Table 3. Immunization of mice against intracranial infection with SHBRV and COSRV

| Vaccine conc., μg | Survivorship* | |
|---|---|---|
| | SHBRV | COSRV |
| 5 | 8/9 | 9/9 |
| 1 | 7/8 | 10/10 |
| 0.2 | 5/9 | 6/10 |
| 0.08 | 4/9 | 3/9 |
| No vaccine | 1/9 | 0/9 |

The $ED_{50}$ values calculated from survivor rates were as described (24); they are as follows: for SHBRV, 0.126 μg; for COSRV, 0.120 μg.
*Groups of 10 mice were immunized i.p. on days 0 and 7 with listed concentration of vaccine and challenged intracranially with 100 MIC $LD_{50}$ of either SHBRV or COSRV. Data are given as no. survivors/total no.

Our *in vivo* results led us to speculate that SHBRV may grow better in fibroblasts or epithelial cells than COSRV. *In vitro* studies revealed that, while the susceptibility of NA cells to infection by both viruses was similar, the infectivity of SHBRV was much higher than that of COSRV in fibroblasts (BHK-21) or epithelial cells (MA-104). Interestingly, incubation temperature was found to have a marked effect on the infectivity of SHBRV in BHK-21 and MA-104 cells, with ≈10 times more cells being infected with a particular dose of virus at 34°C than at 37°C. Low pH-induced fusion and cell-to-cell spread of virus in SHBRV-infected BHK-21 cells was also seen to be limited to 34°C. This temperature effect on infectivity and fusogenic activity of SHBRV could be due to the replacement of the cysteine residue at position 207, resulting in a decreased stability of the G protein at higher temperature.

The low pH-dependent fusion process, which is mediated by the viral G protein (26), is thought to facilitate the internalization of the rabies virus into the host cell (30–33) and may also be responsible for the ability of the virus to spread from cell to cell (25). Our studies revealed that, in contrast to tissue culture adapted rabies virus strains, which can mediate pH-dependent fusion in NA cells as well in BHK cells (34), street viruses preferentially cause fusion in NA cells. In BHK cells pH-dependent fusion can only be mediated by SHBRV and only at 34°C but not 37°C. Furthermore, the pH optimum of fusion activity observed in SHBRV- or COSRV-infected NA cells (pH range of 5.2 to 5.4) appears to be significantly lower than the pH optimum for fusion seen NA cells infected with tissue culture adapted strains (pH range of 5.6 to 5.8) (26). These data demonstrate that street viruses differ considerably from tissue culture-adapted strains in their ability to cause cell fusion, which may be related to the differences seen in the neuropathogenicity of these viruses.

Biological differences between SHBRV and COSRV, apparent in our *in vitro* experiments and studies of infectivity in mice, may be relevant to the transmission of these viruses to humans. The major unifying conclusion is that the epidermis and dermis may provide a portal for the entry of SHBRV during a natural infection. The ability to replicate in epidermal cells at the surface of the body, subject to slightly lower temperatures, may enable a small dose of SHBRV to amplify at the inoculation site, thereby enhancing the probability of finding and penetrating a nerve fiber. Obviously, the amount of virus that can be introduced by the bite or scratch of a small insectivorous bat is negligible compared to the level of exposure due to the bite of a large carnivore, such as a coyote, dog, raccoon, or skunk. SHBRV may have become adapted such that only a small, superficially administered quantity of the virus is sufficient to cause infection and lethal disease. Future studies of isolates of rabies virus originating from silver-haired and other bats will determine whether or not the attributes described above are common to other strains. In light of these findings, we believe that it is prudent to recommend post-exposure vaccination for people who may have come in contact with a bat.

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